

HUMAN RECOMBINANT FACTOR VIIA IS NEUROPROTECTIVE IN A MODEL OF TRAUMATIC BRAIN INJURY AND SECONDARY HYPOXEMIA

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ABSTRACT

Factor VII (FVII) circulates in plasma as a zymogen until it is exposed to tissue factor (TF). When bound to TF, activated FVII (FVIIa) initiates the extrinsic coagulation cascade that results in the formation of a polymerized fibrin clot. In the untraumatized brain, TF is physically isolated from FVII. However, traumatic brain injury (TBI) frequently results in the disruption of the vascular endothelium and resultant exposure of FVII to subendothelial TF. Recent evidence suggests that proinflammatory cytokines (IL-1, IL-6, TNF α) can induce the upregulation of TF. Since these cytokines also have been implicated in the inflammatory response to TBI, it is conceivable that, in the traumatized brain, treatment with recombinant (r) FVIIa might exacerbate injury-induced coagulopathy. In an effort to evaluate this hypothesis, rats were trained to execute a visual discrimination and locate the submerged platform in a Morris water maze (MWM) before being subjected to moderate parasagittal fluid percussion injury (FPI) immediately followed by 30 min of 10% O₂. Ten min before FPI, rats were injected IV with 4 mg/kg of human rFVIIa; clotting and factor assays revealed that this was a hemostatically active dose. Visual discrimination performance was continuously recorded for two weeks after FPI and on day 7 after FPI, retention was tested in the MWM. After a two-week recovery period, brains were perfusion fixed and processed for histological evaluation. Contrary to our expectation, preliminary findings revealed that FPI reduced neuronal cell density and disrupted the pyramidal and granule cells of the dentate gyrus less in rats treated with rFVIIa than in rats treated with vehicle. Furthermore, visual discrimination accuracy was reduced less and recovered more rapidly and the mean retention score was larger for rats treated with rFVIIa than for rats treated with vehicle. Thus, rather than revealing a therapeutic complication, these preliminary preclinical findings agree with recent clinical data and suggest that rFVIIa is neuroprotective.

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1. INTRODUCTION

Exsanguinating hemorrhage is a principal cause of battlefield mortality. As a consequence, hemostasis has been a principal focus of combat casualty care research. Laboratory, clinical, and field studies have successfully demonstrated that a bandage impregnated with fibrin can be used to stop exsanguinating hemorrhage at a wound site. Although quite effective, the fibrin bandage might not be especially useful for treating hemorrhage remote from a wound site nor can it be used to treat dilutional coagulopathy that might result from massive blood replacement therapy. A systemic complement to the fibrin bandage is activated recombinant Factor VII (rFVIIa).

Endogenous Factor VII (FVII) is a serine protease that circulates in plasma in its inactive zymogen form until it is exposed to tissue factor (TF), a membrane-bound protein that while essentially absent on normal resting endothelium, is abundant in the subendothelium, in the adventitial cells that surround blood vessels, and in the brain within astrocytes (Morrissey, 2004). In the absence of trauma, TF is physically isolated from FVII. However, trauma frequently results in the disruption of the vascular endothelium and resultant exposure of FVII to subendothelial TF. Once bound to TF, FVII is activated and, as shown in Figure 1, the TF+FVIIa complex initiates the extrinsic and common coagulation cascades that result in the formation of a polymerized fibrin clot.

One might hypothesize therefore that treatment with rFVIIa might minimize hypotension resulting from trauma-induced hemorrhage. In support of this, Lynn et al. (2002) reported that human recombinant FVIIa significantly minimized the reduction of mean arterial pressure and shortened prothrombin (PT) time in a laboratory study of trauma-induced hemorrhage in pigs.

In humans, hemorrhagic hypotension is frequently a form of secondary trauma that occurs in conjunction with traumatic brain injury (Chesnut, 1993). Consequently, rFVIIa might also be useful for treating the hemorrhage and hypotension that result from traumatic brain injury (TBI).

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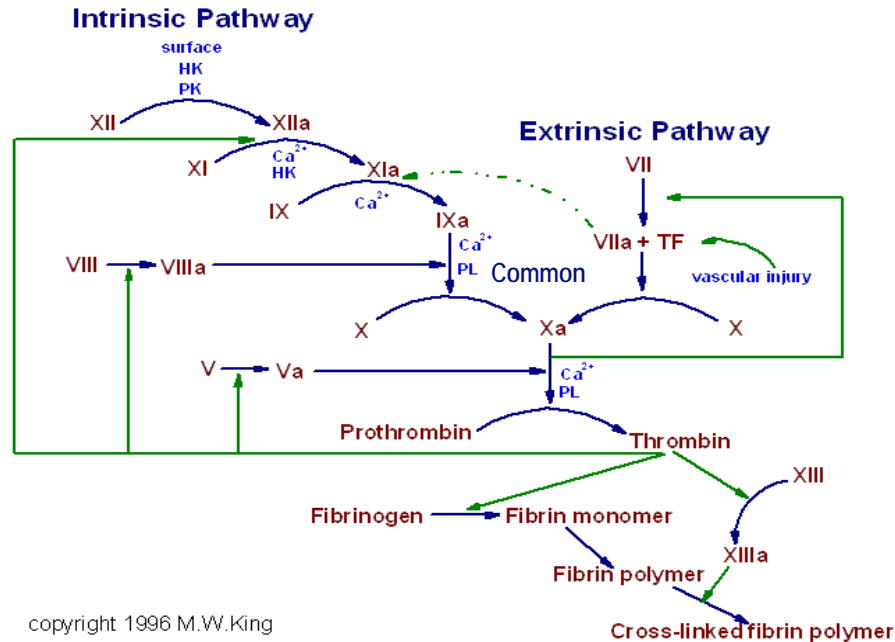


Figure 1. Vascular injury exposes **tissue factor (TF, factor III)** to phospholipid (PL), which results in the activation of FVII (FVIIa). The TF+VIIa complex activates Factor X, which is the common link between the intrinsic and extrinsic pathways. Once activated, FXa hydrolyzes prothrombin to thrombin, which catalyzes the conversion of fibrinogen to fibrin resulting in the formation of a polymerized fibrin clot. Factor XIIIa cross-links fibrin polymers solidifying the clot. HK = high molecular weight kininogen. PK = prekallikrein.

Recent evidence suggests however that proinflammatory cytokines (IL-1, IL-8, and TNF α) can induce the upregulation of TF (Grignani and Maiolo, 2000) and since these same cytokines also have been implicated in the inflammatory response to TBI (Toulmond and Rothwell, 1995; Sanderson, et. al. 1999), it is conceivable that, in the traumatized brain, rFVIIa might exacerbate injury-induced coagulopathy. Moreover, Stein, et. al. (2002) showed that intravascular coagulation is a major secondary insult after fluid percussion injury (FPI) in rats, rotational acceleration injury in pigs, and contusional brain injury in humans. In an effort to evaluate the safety of rFVIIa in a model of traumatic brain injury, rats were subjected to fluid percussion brain injury and treated with rFVIIa or the vehicle. Neuropathological changes and recovery of sensory and memory functions were evaluated for each group.

2. METHODS AND MATERIALS

2.1 Visual discrimination training

Prior to fluid percussion injury (FPI), rats were trained to repeatedly execute a flicker frequency visual

discrimination for their entire daily food ration. When presented with a rapidly flickering cuelight, a press on the left lever resulted in the delivery of a food pellet into a recessed food trough. However, if the cuelight flickered slowly, then a press on a right lever resulted in the delivery of a food pellet.

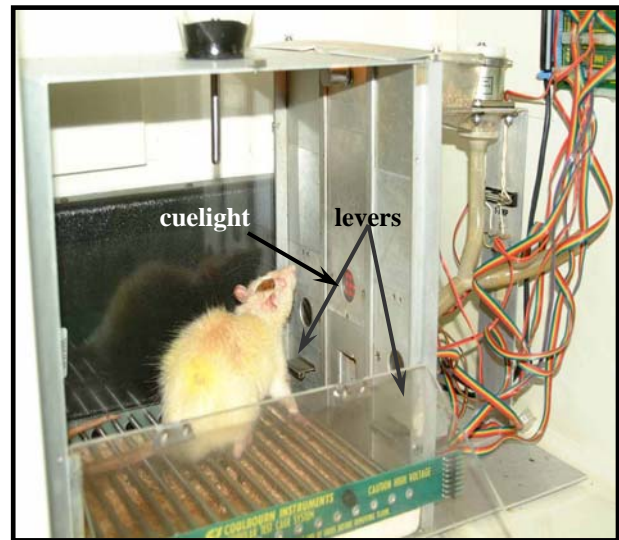


Figure 2. Rat executing the flicker-frequency visual discrimination

Figure 2 shows a rat attending to the flickering red cuelight along the front wall of the test cage. The levers can be seen to either side of the opening to the recessed food trough. After FPI, the slow and the fast flicker frequencies were increased by a constant.

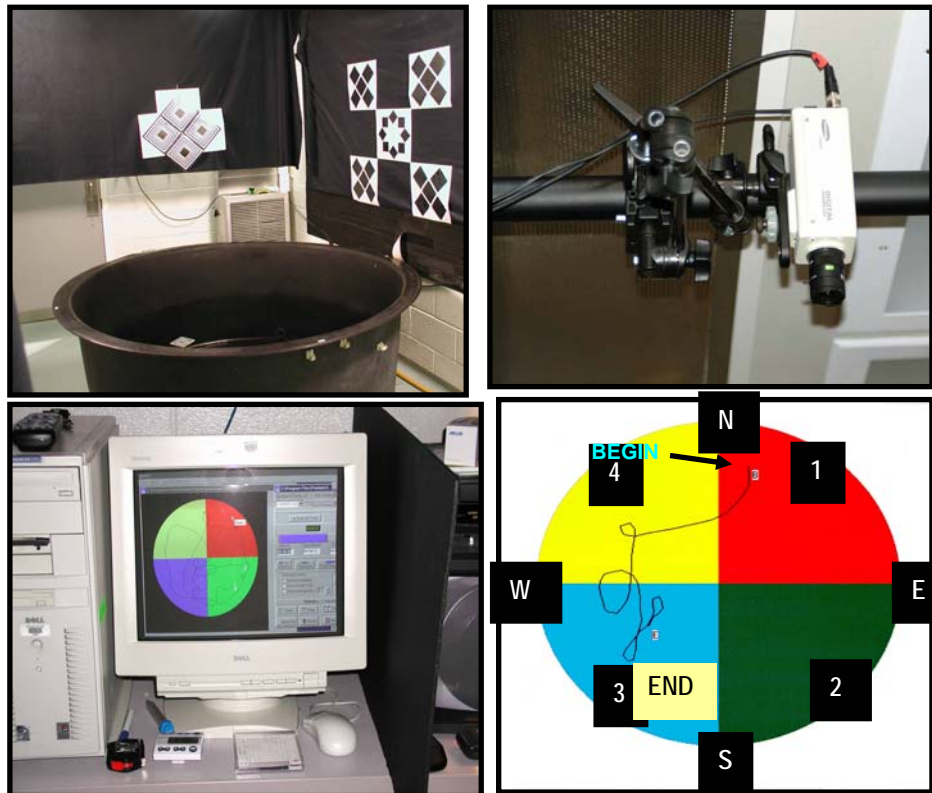


Figure 3. The Morris water maze (MWM) and video tracking system are shown below.

2.2 Morris water maze and Retention Testing

One week before surgical preparation, each rat was trained to find the submerged platform in the SW quadrant of a 176cm pool (top left panel in Figure 3) from any one of the four cardinal compass locations. A computerized video-tracking system (top right and bottom left) was used to record swimming time and distance. The four quadrants, release locations, and a sample track are shown in the bottom right panel.

On the seventh day after FPI and hypoxia, memory retention was measured. The platform was removed from the SW quadrant and individual rats were allowed to swim for 60 sec in the MWM. The video-tracking program recorded the amount of time spent in the SW quadrant.

2.3 Surgical preparation

After visual discrimination and MWM training, individual rats were injected IM with a mixture of ketamine and xylazine (70 and 6 mg/kg, respectively), shaved, mounted in stereotaxic frame, and lidocaine was applied to the scalp. A midline incision was made, the scalp was reflected, the surface of the cranium was

cleaned, and a trephine was used to remove a 4mm flap of skull above the right parasagittal cortex midway between Bregma and Lambda. The hub of a 1cc hypodermic needle was glued into the opening, skull screws were installed rostral and caudal to the hub, and a layer of dental resin was applied to the entire surface of the skull.

2.4 Fluid Percussion Injury, Retention testing, and Factor Assays

Under halothane anesthesia, individual rats were mounted in a stereotaxic frame, fitted with a nose cone that supplied a mixture of compressed air (21% O₂) and 3% halothane, arterial and femoral venous catheters were installed, and each rat was connected to the fluid percussion device, as shown in Figure 4.

Ten min before FPI, rats were injected IV with either 4 mg/kg rFVIIa (n=7) or an equivalent volume of 0.9% NaCl, the vehicle (n=11). All rats were subjected to FPI. Immediately after FPI, the O₂ concentration of the compressed air was switched to 10% for 30 min, at the end of which time PaO₂ averaged 35 mmHg. At the end of the hypoxic period, each rat was returned to its cage and visual discrimination accuracy was monitored for 14 days.

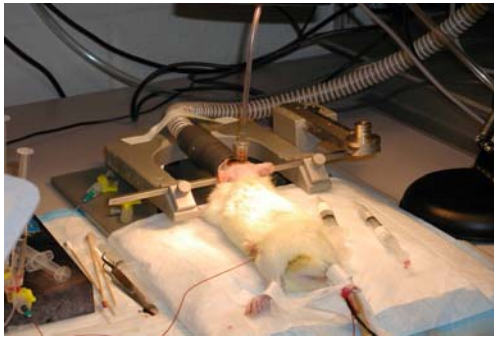
FEMORAL CATHETERS



FPI DEVICE



RAT CONNECTED TO FPI DEVICE



STORED WAVEFORM

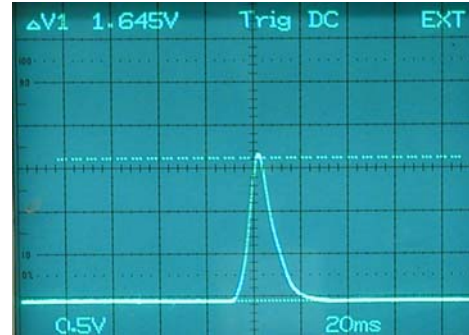


Figure 4. Surgical procedures, injury device, and sample injury waveform.

On the seventh day after FPI, retention was measured. The platform was removed from the SE quadrant and individual rats were allowed to swim for 60 sec in the MWM. The video-tracking program recorded the amount of time spent in the SE quadrant.

At 15 min before and 5 and 120 min after FPI, blood samples were drawn and centrifuged for 25 min at 6,000 rpm, after which the plasma was removed for analysis of clotting times and clotting factor activities. Automated assays were carried using a STA Compact analyzer and included the Prothrombin Time (PT), Activated Partial Thromboplastin Time (aPTT), Thrombin Time (TT), and Fibrinogen. Samples were tested for the activities of clotting factors V, VII, VIII, IX, and X.

2.5 Neurohistology

On the 15th days after FPI and hypoxia, surviving rats from all groups were exsanguinated with saline, and perfused transcardially with a solution of 10% formalin, phosphate buffer, and saline. The brains were removed and stored in 10% formalin until they were shipped to FD NeuroTechnologies (Baltimore, MD) where they were rapidly frozen and sectioned coronally at 40 μ beginning at -2.0B. Pairs of sections were cut 400 μ m apart and alternate sections within a pair were stained with thionine and silver.

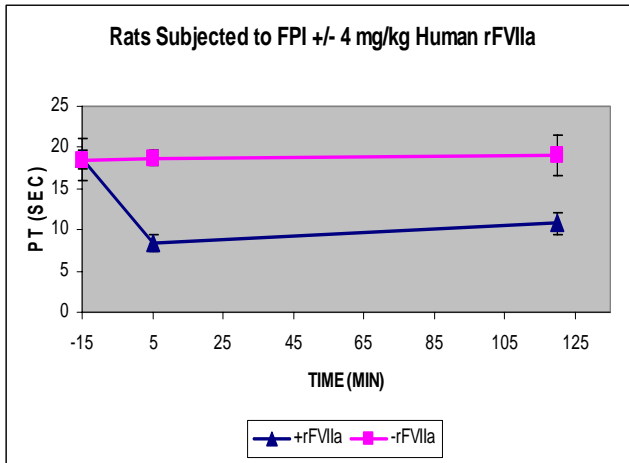
(Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition.)

3. RESULTS

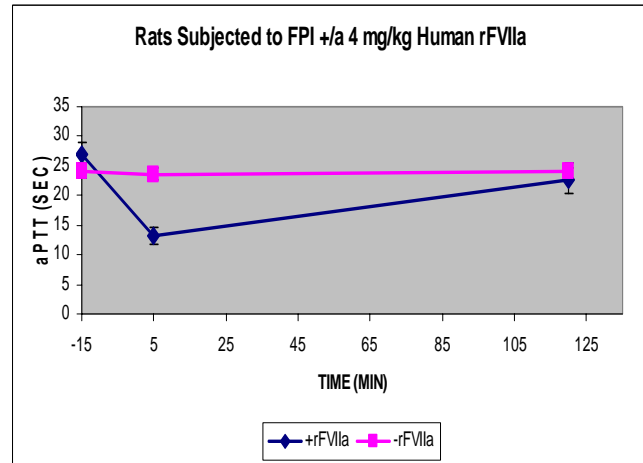
3.1 Clotting Factors

The panels in Figure 5 show that rFVIIa significantly activated the intrinsic, extrinsic, and common coagulation pathways. Prothrombin time (PT) is a measure of the activity of the extrinsic and common pathways and the activated Partial Thromboplastin Time (aPTT) is a measure of the activity of the intrinsic and common pathways. Within the intrinsic pathway, Factor IX, a Vitamin K dependent proenzyme containing carboxylglutamate residues, and Factor V (not shown) were significantly elevated at 5 min after FPI and hypoxia. However, as shown in Figure 1, FVIIa can also activate Factor IX (see the dotted green line in Figure 1). FXa, a serine protease that is activated by both FIXa and the TF+FVIIa complex in the presence of phospholipids and Ca^{2+} , was significantly elevated at 5 and 120 min after FPI and hypoxia. As shown in Figure 1, FXa can also further the activation of FVII.

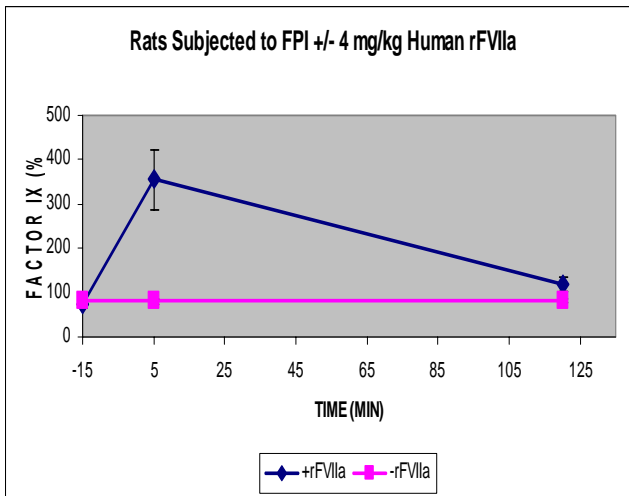
rFVIIa significantly reduces Prothrombin Time at 5 and 120 min after the delivery of rFVIIa.



rFVIIa significantly reduces activated Partial Thromboplastin Time at 5 min after the delivery of rFVIIa.



Five min after the delivery of rFVIIa Factor IX activity is significantly elevated.



Five min and 120 min after the delivery of rFVIIa Factor X activity is significantly elevated

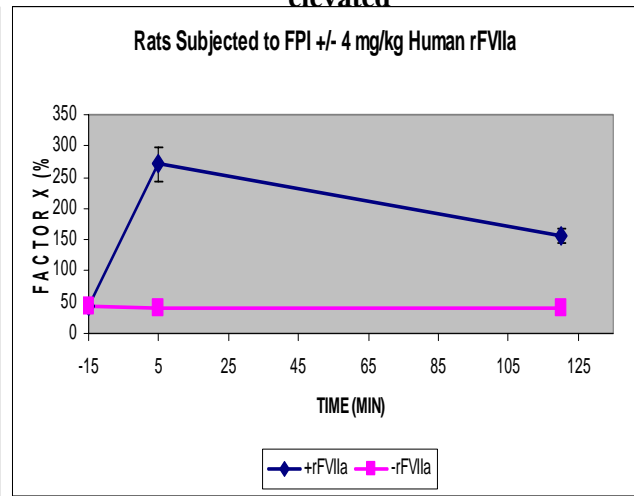


Figure 5. Prothrombin time (PT), activated Partial Thromboplastin Time (aPTT), Factor IX and Factor X activity 15 min before FPI and at 5 and 120 min FPI and secondary hypoxia.

3.2 Neurohistology

Figures 6, 7, and 8 are serial reconstructions of the hippocampal formation ipsilateral and contralateral to the site of injury. Since a 10X objective supplied the minimum necessary magnification for identifying neuronal cell loss in the hippocampus and only a small fraction of the ipsilateral or contralateral hippocampus could be captured in any one digital image, it was necessary to stitch together many serial images to capture the entire hippocampus at each of the levels represented in these figures.

Figure 6 shows the ipsilateral and contralateral hippocampus at -5.8B, a caudal location near the cortical impact site of the fluid pulse, for a rat that was treated with the vehicle. The ipsilateral hippocampus shows that FPI+secondary hypoxia disrupts the neuronal integrity of the granule cell layer and the CA3 field of pyramidal cells. No such injury is evident in the contralateral hippocampus.

Figure 7 shows that pretreatment with rFVIIa significantly minimizes the FPI and hypoxia-induced loss of pyramidal cells in the CA3 field and granule cells in the dentate gyrus.

Figure 8 show that pretreatment with rFVIIa significantly reduces neuronal cell loss in the dentate gyrus at -6.3B.

Figure 6. Hippocampal formation at -5.8B ipsilateral and contralateral to the site of injury for a rat that was treated with the vehicle.

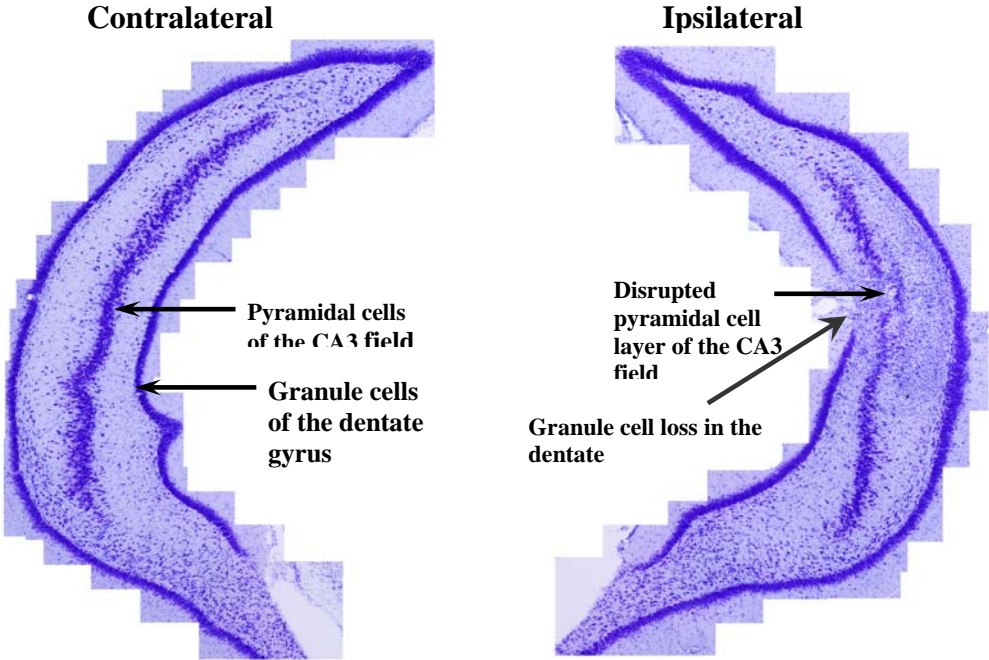
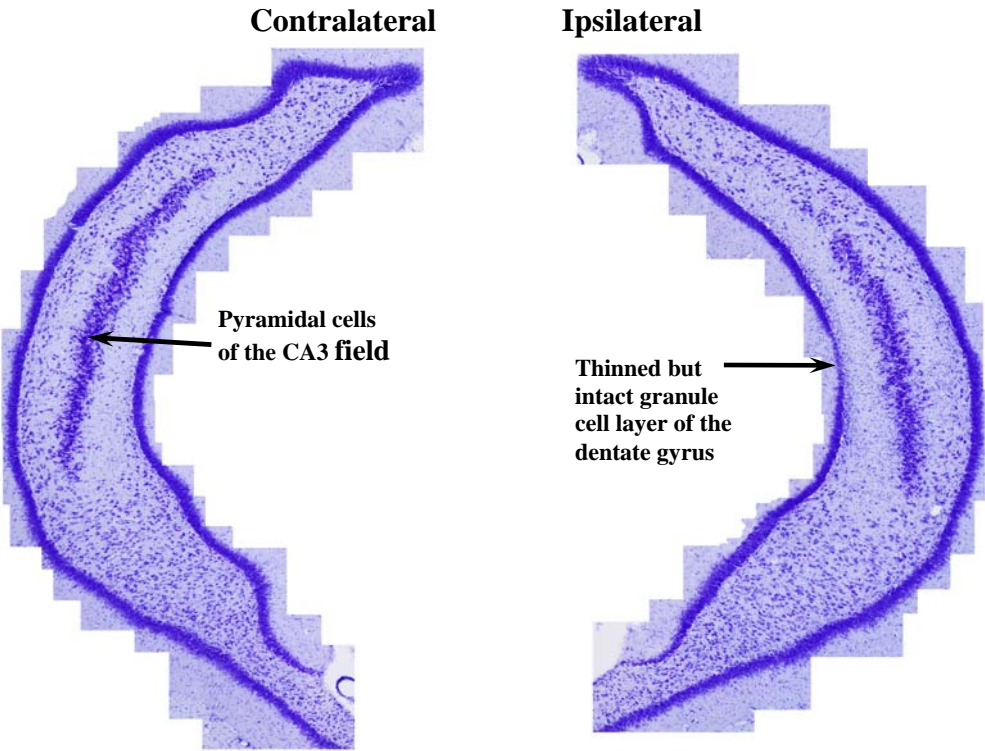


Figure 7. Pretreatment with rFVIIa minimizes neuronal cell loss in the granule cell layer of the dentate and the CA3 field of the hippocampus at 5.8B.



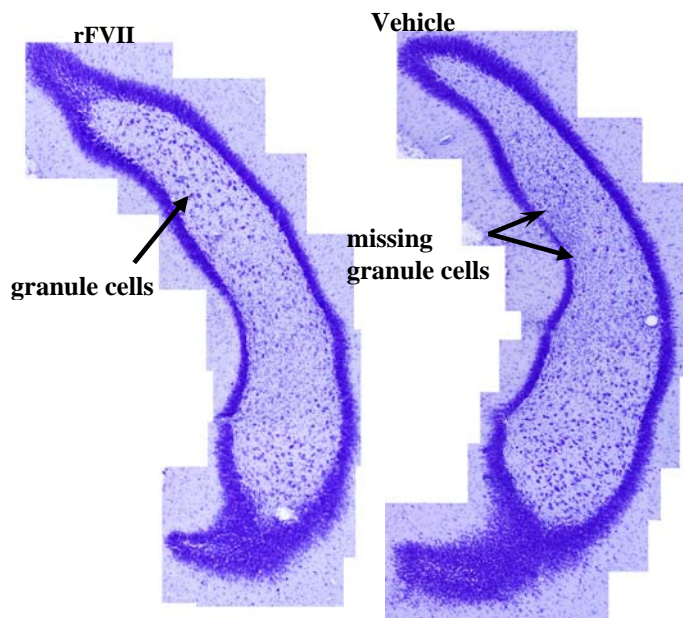


Figure 8. rFVIIa reduces neuronal cell loss in the dentate gyrus at -6.3B.

3.3 Visual Discrimination

Figure 9 shows visual discrimination accuracy for the rFVIIa and vehicle-treated injury groups and for a representative sham group. This sham group was used as a control for a different experiment and with the exception of the injury, was treated identically to the injury groups in the current experiment. Visual discrimination accuracy was reduced less and recovered more rapidly for the injury group treated with rFVIIa than for the injury group treated with vehicle.

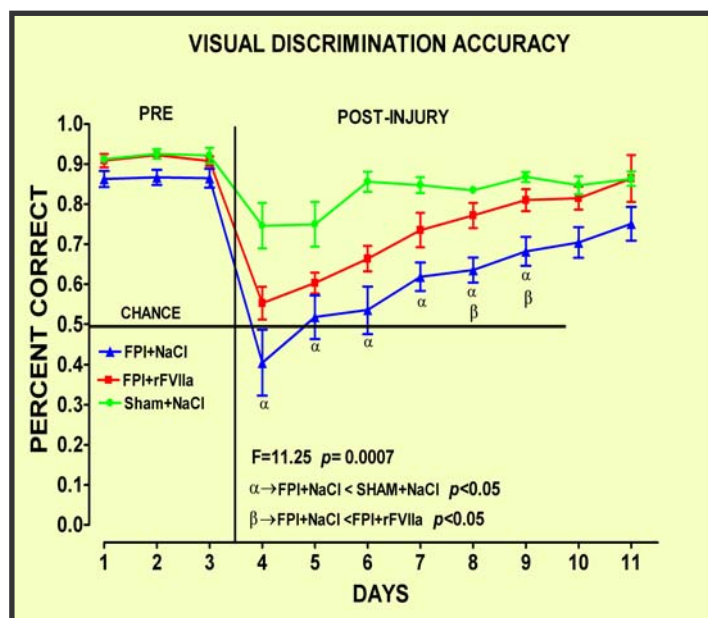


Figure 9. Visual discrimination accuracy for three days preinjury and 8 days post-injury for both injury groups and a sham injured group.

3.4 Morris Water Maze

Figure 10 shows that rFVIIa increased the time spent in the quadrant within which the platform was located.

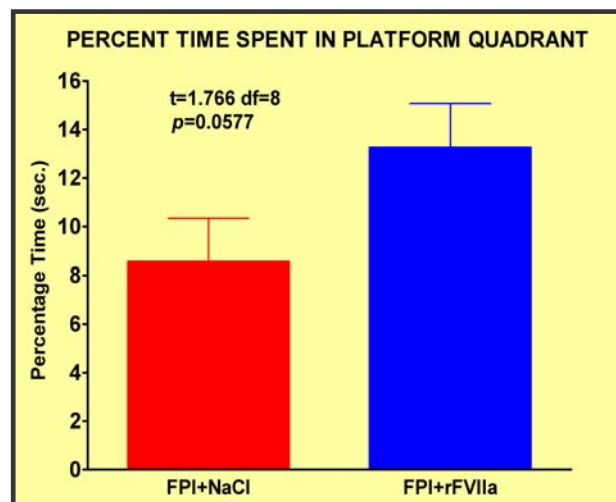


Figure 10. In the test for retention, rFVIIa increased the time spent in the (SE) platform quadrant.

4. CONCLUSIONS

The delivery of rFVIIa clearly activated the intrinsic, extrinsic and common coagulation pathways. The factor assays revealed that Prothrombin Time (PT), a measure of the integrity of the extrinsic and common pathways of coagulation, and activated Partial Thromboplastin Time (aPTT), a measure of the integrity of the intrinsic and common pathways of coagulation, were significantly reduced at 5 min and 125 min after the delivery of rFVIIa, although fibrinogen formation was not significantly increased at either time point (not shown). FX activity was also significantly elevated. In the absence of rFVIIa, FX is activated on the surface of activated platelets by the tenase complex, $\text{Ca}^{2+} + \text{FIXa} + \text{FVIIIa} + \text{FX}$. This complex converts FX to FXa, which is necessary for clot formation. However, the addition of 4 mg/kg of rFVIIa is approximately three orders of magnitude larger than the frequently used therapeutic dose of approximately 100 micrograms/kg. Consequently, it is possible that the massive quantities of FVIIa in the systemic circulation could have initiated the activation of Xa via the intrinsic pathway (see figure in introduction). Most interesting is that FVIIa was significantly elevated at 5 min and 2 hours. However, FIXa, a factor within the intrinsic pathway, was elevated at only 5 min after the delivery of rFVIIa. This discrepancy suggests that perhaps the abundantly available FVIIa in a traumatized brain could have directly activated FX via the VIIa+TF complex.

Whatever the reason for the significant increase in FXa, rFVIIa was not expected to be neuroprotective and perhaps with the collection of additional data the conclusion will be that it is in fact not neuroprotective. However, the preliminary data that are presented here leads one to a very different conclusion. rFVIIa protected the hippocampus from the secondary consequences of experimental traumatic brain injury and this protection appears to translate into a very robust and lasting recovery of visual discrimination accuracy. Specifically, in a visual discrimination test that required rats to learn to press levers in spatially distinct locations when presented with either of two different flicker frequencies, the asymptotic level of discrimination accuracy was reduced less and recovered more rapidly for those rats that were experimentally brain injured and received rFVIIa than for those rats that were brain injured and received the vehicle. It should be emphasized that although the visual discrimination accuracy for the rFVIIa treated rats was not always significantly greater than the visual discrimination accuracy of vehicle-treated rats, it was consistently greater and recovered more rapidly. Perhaps the protection afforded the hippocampus, the neuronal integrity of which has been frequently implicated in the control of spatial learning and performance, is in part important for explaining the visual discrimination findings because a critical feature of the visual discrimination test required leverpresses in different spatial locations. The greater retention time for the rats treated with rFVIIa in the MWM, a test of spatial memory, is consistent with this possibility. Taken together, the results from the visual discrimination test and the MWM test offer convergent confirmation of the neuroprotective efficacy of rFVIIa in a model of severe traumatic brain injury.

One might legitimately ask what the reason might be for the reduction of injury to the hippocampus and the relatively greater visual discrimination accuracy and retention in the rFVIIa-treated rats. It is clear from laboratory and clinical findings that rFVIIa can be used to treat a subgroup of hemophilia patients who develop antibodies, or inhibitors, to traditional hemophilia treatments. In fact, the FDA has approved the use of rFVIIa for this purpose. There are also published findings that support the efficacy of rFVIIa as a treatment for coagulopathic abnormalities in neurosurgical patients that were treated with anticoagulant medication and suffered from end-stage cirrhotic disease and traumatic brain injury. Perhaps one reason why rFVIIa was neuroprotective in the present study was that it reduced intracranial hemorrhage. Although preliminary, support for this can be found in Long, Bauman, et. al. (2004), who reported that FPI results in intraparenchymal hemorrhage ipsilateral to the site of injury.

Traumatic brain injury on the battlefield frequently results in intraparenchymal hemorrhage and hemorrhagic hypotension. Consequently, it is conceivable that rFVIIa might have considerable clinical value to the combat medic who must treat traumatically brain-injured warfighters. In particular, rFVIIa could be used to minimize increased intracranial pressure, which, if untreated, invariably results in increased morbidity and mortality. As a consequence, more laboratory data needs to be collected to confirm the findings of the present study at the same time that clinical trials are planned that would target the hemorrhaged head-injured combat casualty for treatment with rFVIIa.

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